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Structural inflexibility of the rhizosphere microbiome in mangrove plant *Kandelia obovata* under elevated CO_2

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ABSTRACT

Rhizosphere microbial communities play an important role in mediating the decomposition of soil organic matter. Increased CO_2 concentration may increase plant growth by stimulating photosynthesis or improving water use efficiency. However, possible eco-physiological influences of this greenhouse gas in mangrove plants are not well understood, especially how rhizosphere microbial communities respond to CO_2 increase. We characterized the effect of elevated CO_2 (eCO_2) on rhizospheric microbial communities associated with the mangrove plant *Kandelia candel* for 20 weeks, eCO_2 increased plant chlorophyll *a* levels and root microbial biomass. Operational taxonomic unit analysis revealed no significant effects of eCO_2 on rhizospheric bacterial communities; however, some influence on archaeal community structure was observed, especially on the ammonia-oxidizing archaea. Principal component analysis showed that microbial biomass C, total nitrogen, C/N ratio, nitrate nitrogen, and salinity were the main factors structuring the microbial community. The relative contribution of environmental parameters to variability among samples was 31.0%. In addition, functional analysis by average well color development showed that carbon source utilization under eCO_2 occurred in the order amino acids > carbohydrates > polymers > carboxylic acids > amines > phenolic acids; whereas, sugars, amino acids, and carboxylic acids were the preferred carbon sources in control groups. Differences in utilization ability of carbohydrates and amino acids resulted in changes in carbon metabolism between the two groups. Rhizosphere microbial communities appear to have some buffering ability in response to short-term (20 weeks) CO_2 increase, during which the metabolic efficiency of carbon sources is changed. The results will help better understand the structural inflexibility and functional plasticity of the rhizosphere microbiome in mangrove plants facing a changing environment (such as global climate change).

1. Introduction

Carbon dioxide concentration in the atmosphere has increased about 21% from 280 parts per million (ppm) in preindustrial times to approximately 390 ppm today and is predicted by some models to double within the next century (Black et al., 2011). Current estimates suggest that the atmospheric CO_2 concentration range will lie between 450 ppm and 600 ppm by the year 2150 (Barrios et al., 2012). Increases in CO_2 can contribute to global warming, which may have a direct impact on plant growth and development by stimulating photosynthesis or improving water use efficiency. As elevated CO_2 (eCO_2) would increase carbon supply to soil sediment (Loiseau and Soussana, 1999), studies on the effects of increased atmospheric CO_2 in a land ecosystem may yield valuable information to better understand its feedback to global climate change. In particular, as plants are essential for the recycling of materials in natural ecosystems, it is important to study the impacts of eCO_2 on various kinds of plant ecosystems, including the wetland niche.

Mangroves are among some of the most diverse and highly productive coastal ecosystems in tropical and subtropical regions (Bhattacharyya et al., 2015). Wetland plants contain dense and abundant microbial communities within the thin layer of root-adherent soil known as the rhizosphere environment (Bulgarelli et al., 2013). Rhizospheric bacteria live in the direct vicinity of the roots and play important roles in mangrove ecosystems, including nitrogen fixation (Alfaro et al., 2015), nutrient acquisition (Holguin et al., 2001; Bakker,

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2012; Jayaprakash et al., 2015), abiotic stress tolerance (Rout and Southworth, 2013) as well as production of regulators of plant growth and development such as auxins, cytokinins, and gibberellins (Ahmad et al., 2013). Additionally, the microbial communities associated with roots also play an essential role in the cycling of matter (for instance phosphorus, organic acids, and siderophores) and maintenance of the health of wetland ecosystems (Gomes et al., 2011; Zeng et al., 2014).

Several factors have been identified that influence microbial communities, including global climate changes and eCO_2. Based on the important relationship between plants and microbes, therefore, it is necessary to examine the diversity, composition, and structure of microbial communities and their links with environmental factors for improving our understanding of mangrove ecosystem functioning. In past decade(s), many studies have made attempts to observe how rhizosphere microorganisms respond to changes in CO_2. However, the results have been inconsistent. Due to the complex interactions with biotic and abiotic factors, the responses of microbial communities to eCO_2 are still poorly understood and contradictory. Some studies have revealed changes in microbial community composition under elevated CO_2 (Hodge et al., 1998; Hayden et al., 2012), while others have shown no significant differences (Klamer et al., 2002; Grüter et al., 2006; Kanerva et al., 2008). Studies on the effects of eCO_2 on microbial biomass, metabolic activity, and total community patterns have documented a variety of responses from significant changes to no response (Grüter et al., 2006; Hayden et al., 2012; Terrer et al., 2018). These differences may be related to plant type, niche conditions, and the sensitivity of methodologies (Luna-Vega et al., 2012; Wang et al., 2014). This has led to the suggestion that soil microbial communities still hold many mysteries due to their extraordinary complexity. Our understanding of the effects of elevated atmospheric CO_2 on plant-soil interactions is still incomplete. Thus, it is necessary to determine how the bacterial community structures and related functions in wetland ecosystem will respond to eCO_2.

To address these issues, we took advantage of a short-term (20 weeks) CO_2 enrichment experiment under laboratory conditions. We hypothesized that increasing CO_2 would alter the composition and function of rhizosphere microbes. To test the hypothesis, the effect of eCO_2 on the activity and structure of the microbial community associated with *Kandelia obovata*, a dominant, important local species in the south of China, was investigated. The aim was to better understand the potential effects that global climate change might have on soil microbial communities. In addition, the habitat of *K. obovata* lies in the southern coastal area of China, which is influenced by the dual factors of human activities and climate change. Improved understanding of the microbial response to eCO_2 perturbations may potentially enable predictions to be made regarding how complex wetland microbial communities may be affected by future anthropogenic changes.

2. Materials and methods

2.1. Experimental design

Six independent chambers were constructed (Fig. 1). Young mangrove individuals (plant height 33.1 ± 2.4 cm, and diameter at breast height 0.96 ± 0.14 cm) were transferred to chambers containing soil collected from a field in the Leizhuo Peninsula (south coastal region of China) that was dominated by *K. obovata*. Prior to use, the soil was homogenized by thorough hand mixing while wearing sterile gloves and sieved. The plants were arranged into six chambers (three treatment groups and three control groups) each containing seven individual pots. The plants were cultured under laboratory conditions for 20 weeks: (i) control (aCO_2; incubation at 350 ppm ambient CO_2); (ii) eCO_2 (incubation at 700 ppm elevated CO_2).

In cubation parameters were automated by continuous monitoring during the experimental process. No fertilizer was added throughout the experiment, and all groups were treated with the same temperature and humidity. The chambers were maintained at 28 °C and 500 μE/m^2/s of light on a 12 h light/dark cycle. Natural brackish seawater with a salinity of 14.1‰ was used every day for plant submergence (Chaudhary et al., 1974). The water depth was kept 5 cm above the soil surface, and water levels in the containers were checked weekly and were adjusted with distilled water to compensate for evapotranspiration losses. Each aquarium was covered with a custom clear acrylic lid with neoprene seals that was secured to the top of the tank with bungee cords. The lid contained two gas port fittings and one temperature probe. Automatic monitoring and control of CO_2 flux within the chambers were accomplished using electronic input/output hardware and programmable software. To monitor CO_2 levels, an infrared CO_2 gas analyzer was used in each chamber. Each of the chambers was connected with two 1/8 internal diameter Teflon tubes to the gas solenoids. CO_2 and temperature readings for each chamber were saved to a local data server. When the CO_2 level was less than the set point, the CO_2 gas solenoid opened for 1 s with 99% CO_2 gas at 20 kPa for each chamber independently. This “round robin” sampling method was continuous throughout the 20-week exposure period. Fig. 1A shows the schematic diagram of the system; and Fig. 1B shows the CO_2 concentration curve during the whole experimental process.

2.2. Plant parameters

Sampling and index measurement were conducted according to time series. Samples were taken at seven different time-point (i.e. 0^{th}, 1st, 3rd, 5th, 9th, 13th, and 20th week) for twenty weeks, and three plant individual were collected at each time. Collection of roots and leaves was made according to described methods (Paula-Freire et al., 2013). Leaf area was detected by a scanner for scanning and conversion. Chlorophyll *a* (ChII *a*) was extracted by using 95% acetone-ethanol. The absorption of the extract was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Briefly, leaves were washed with water, and absorbent paper was used to blot dry surface moisture; the main vein was removed, and the leaf was cut into strips (about 1–2 mm); 0.5 g leaf (weighed with an accuracy of 0.1 mg) was put into a 50 mL colorimetric tube, and 25 mL extract was added (leaf direct immersion method). Absorbance at 645 nm and 663 nm was measured after 48 h using Arnon's formula (Almeselmani et al., 2011): ChII *a* concentration (μg/L): $C_a = 2.7A_{663}\text{-}2.69A_{645}$; ChII *b* concentration (μg/L): $C_b = 22.9A_{645}\text{-}4.68A_{663}$; total ChII concentration: $C_{a+b} = C_a + C_b$. The ChII concentration of the extract was calculated and converted to ChII content per gram of fresh leaves (μg/g fresh leave weight) (Lin et al., 2015).

2.3. Soil physicochemical parameters

At each sampling time-point, the rhizosphere soil was collected from three representative pots using a sterile blade. We manually removed soil adhering to the roots using spatulas. The rhizosphere soil was homogenized, frozen at −80 °C, and lyophilized prior to analysis. Homogenized subsamples were collected for determination of total C (TC), total N (TN), microbial biomass C (MBC), and dissolved inorganic N (NH_4^+ and NO_3^-). The methods were as described previously (Adam et al., 2000). Briefly, soil pH was determined using a fresh soil-to-water ratio of 1:5 (pH meter). The total soil C and N content were determined by combustion using a TC-TN analyzer (CNS-2000; LECO, MI, USA). The soil nitrate (NO_3^-) and ammonium (NH_4^+) were prepared by adding deionized water (9 mL) to soil (1 g) and shaking for 10 min. After centrifuging at 10,000 rpm for 5 min, the samples were passed through a 0.45-μm filter and frozen until analysis. Soil nitrate was measured using a nitrate electrode (Zhen et al., 1991). NH_4^+ concentrations were measured using the indophenol blue method (Mantoura and Woodward, 1983) with a DR 2000 Autoanalyzer at 425 nm. MBC was determined using the chloroform fumigation direct extraction technique (Brookes et al., 1985). Briefly, 5 g of homogenized,

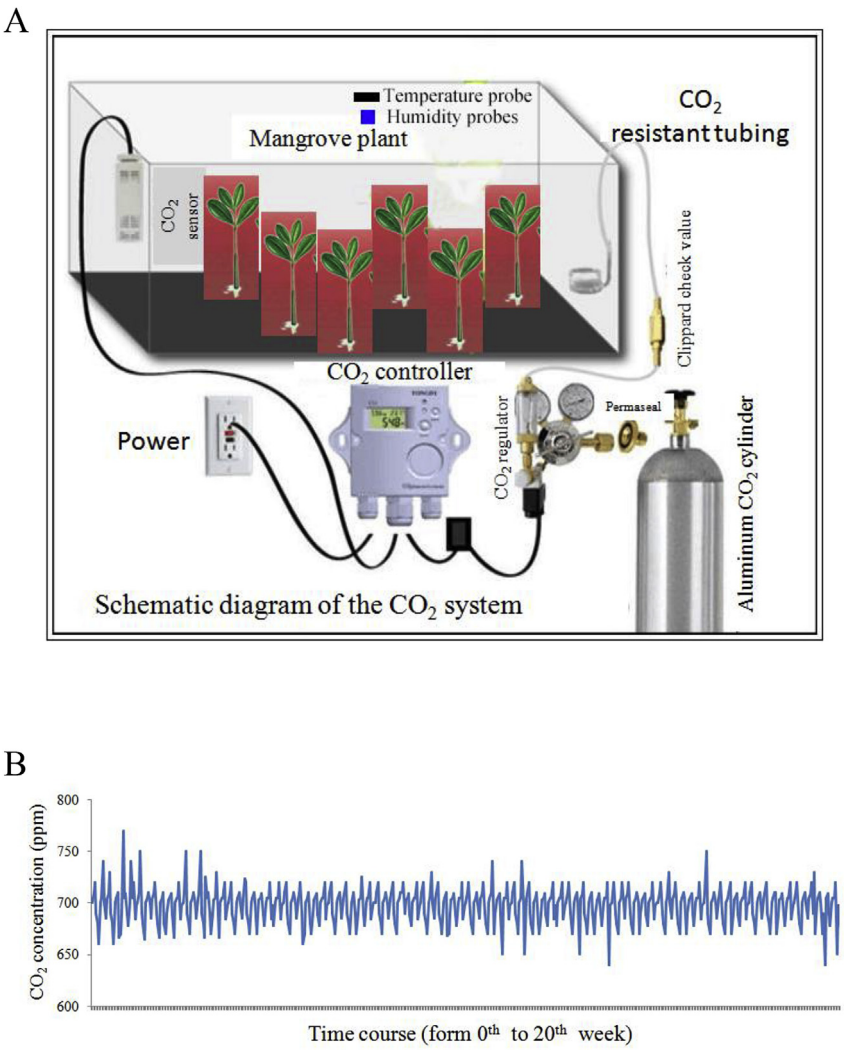

Fig. 1. The schematic diagram of the experimental system (A), and CO_2 concentration curve during the whole experimental process (B).

field-moist soil was fumigated in a vacuum desiccator using 25 mL of ethanol-free chloroform. We evacuated and vented the fumigation chamber four times prior to sealing it for 48 h. Fumigated soils were extracted and filtered, then the extracts for MBC determination were analyzed on a Shimadzu TOC analyzer (Shimadzu, Kyoto, Japan) after acidification with phosphoric acid. A multi-point calibration curve was constructed using potassium phthalate as a standard. MBC was calculated as the difference in TOC extracted from fumigated and unfumigated soils.

2.4. DNA extraction and PCR amplification

From about 0.5 g of each soil sample, total DNA was extracted using an UltraClean Soil DNA Isolation Kit (MoBio, USA), as specified by the manufacturer. DNA samples were amplified by PCR using the fluorescently labeled forward primer 27 F (5′-[6FAM]-AGAGTTTGATCCTGG CTCAG-3′) and the unlabeled reverse primer 927R (5′-CCGTCAATTC-CTTTRAGTTT-3′), which target bacterial 16S rRNA genes (Mummey and Stahl, 2003). For the archaeal community, PCR was performed using the unlabeled forward primer Arch109F (5′-ACKGCTCAGTAAC-ACGT-3′) and the fluorescently labeled reverse primer Arch915R (5′-[NED]-GTGCTCCCCCGCCA ATTCCT-3′), which target the archaeal 16S rRNA gene (Fisk et al., 2013). Amplifications were carried out in triplicate 50 μL PCR reactions comprising 0.5 μM forward and reverse primers, 1 × GoTaq Flexi Reaction Buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 200 mM dNTPs, 0.8 μg/mL BSA, 2.5 U of GoTaq Flexi DNA Polymerase (Promega), and 10 ng of total DNA. The PCR cycling conditions were as follows: 2 min at 94 °C; 10 cycles of 94 °C for 30 s, 65 °C for 30 s (decreasing 1 °C per cycle for 30 s); 18 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension of 72 °C for 10 min.

All PCR products were pooled, cleaned, and concentrated using a QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA, USA). A single composite sample for pyrosequencing was prepared by combining approximately equimolar amounts of PCR products from each sample. Sequencing was carried out at Biolinker Biotech. Co. Ltd. (Shanghai, China).

2.5. Bioinformatics analysis

Raw sequences were processed and checked using Mothur and QIIMe software packages (Schloss et al., 2009). Sequencing data were denoised using the commands “shhh.flows” (Hansen and Klaenhammer, 2011) and “pre.cluster” (Majaneva et al., 2015) on the Mothur platform. Chimeric sequences were identified and removed using UCHIME with the de novo method (Penton et al., 2013). The raw sequence reads were trimmed and filtered according to a previously described method (Perry et al., 2016). Poor-quality sequences (i.e., sequences of 200 bp with an average quality score of 25 and ambiguous characters) were discarded. After low-quality reads were removed, representative

sequences were annotated using an alignment tool (BLAST) against the Ribosomal Database Project and the Silva database augmented with sequences from major wetland taxa (for 16S rRNA sequences). We assigned operational taxonomic units (OTUs) at a 3% gene identity threshold using the UPARSE (version 7.1; http://drive5.com/uparse/) pipeline (Edgar, 2013).

2.6. Carbon source utilization pattern analysis of rhizosphere microbes in K. candel

The carbon metabolism profile of rhizosphere microbes was assessed using the Biolog EcoPlate™ (Biolog, Inc., USA), which contains 31 carbon sources representing six groups of compounds (carbohydrates, amino acids, carboxylic acids, polymers, phenolic acids, and amines) (Zak et al., 1994) and one water blank with three replicates. The soil suspension eluted from the roots of *K. candel* was diluted approximately 1000 times, according to the Biolog Ecoplate requirements, and 150 μL of the diluted soil suspension was added to each well. The Eco-plate was then placed at a constant temperature of 25 °C. The absorbance at 595 nm was measured every 12 h for 7–10 days. Purple color development was followed by reading the optical density (OD) at 595 nm using a microplate reader (Infinite F200 PRO, Austria). All substrate readings were corrected using wells with no substrate, and the value in each well was obtained by subtracting the control values from the 595 nm values. Negative OD values after correction were set to zero (Garland et al., 1997). The average well color development (AWCD) was calculated as follows: AWCD = Σ311(C-R)/31, where C is the OD at 595 nm in each carbon source well and R is the OD of the control. The rate of change of the AWCD during the incubation period was assessed. The experiments were performed independently in triplicate.

2.7. Statistical analyses

To evaluate the effects of CO_2 concentration on soil and plants, the biogeochemical factors investigated for each sample were analyzed with a one-way ANOVA and correlation analysis using SPSS version 13.00 (SPSS Inc., USA). Principal component analysis (PCA) was used to characterize microbial community variation according to time and selected environmental variables, and canonical correspondence analysis (CCA) was used to link variations in microbial communities to environmental properties. Both analyses were carried out using Canoco 5 software. A variation partitioning analysis (VPA) was conducted to examine the contribution of environmental factors in influencing microbial community structure as determined by CCA analysis. Significant differences were defined as a $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Plant physiological responses

During the experimental process, *K. obovata* response parameters (ChII *a*, leaf area, shoot mass, and root biomass) were detected at different timepoints (0, 1, 3, 5, 9, 13, and 20 weeks). A significant increase in ChII *a* concentration ($P < 0.05$) was observed from the 5th week. The maximum value was obtained at the end-point, which was increased nearly 9 times compared with the control (Fig. 2A). Similar results were observed for leaf area (cm^2); the degree of increase ranged from 20% to 30% in the eCO_2 group from the 9th week ($P < 0.05$) (Fig. 2B). Shoot mass was also increased by 11.4–13.9% ($P < 0.05$) under eCO_2 conditions compared with the control group (Fig. 2C). The gradual eCO_2 treatment stimulated shoot growth in this mangrove plant species. However, the root biomass was not significantly affected by CO_2 (Fig. 2D) ($P > 0.05$).

3.2. Soil biogeochemical characters

Soil biochemical factors are shown in Table 1, including pH, salinity, NH_4^+, NO_3^-, TC, TN, C/N ratio, and microbial biomass C (MBC). We observed a gradual increase in pH value with time, most notably in the 9th week. However, no significant difference between the treatment group and the control group was observed (Table 1) ($P > 0.05$). Salinity was also not influenced by eCO_2, and the value ranged from 11.7 ± 0.99 to 14.5 ± 1.04 (PSU). Concentrations of NH_4^+ and NO_3^- under eCO_2 increased from the beginning of the experiment to the end of the 1st week by 2.36 and 1.38 times compared with the control, respectively; then, these two factors decreased over the course of the experiment. However, NH_4^+ and NO_3^- concentrations did not show obvious changes between aCO_2 and eCO_2 at the same time-point. Similarly, we did not observe significant variation in soil TC and TN in response to eCO_2. MBC was increased with time and also improved by eCO_2, but small fluctuations existed in the middle stages of the experiment (3^{rd}-5th weeks). The rise in MBC with increasing CO_2 was primarily due to the addition of plant C inputs. However, the C/N ratio remained relative stable throughout the experimental period.

3.3. Biodiversity of rhizosphere microbes

After preprocessing of all reads, 32,104 and 29,175 high-quality sequences were obtained for aCO_2 and eCO_2 samples, respectively. The numbers of sequences from different samples varied in the range of 1654–2987 for aCO_2 samples, and 1513 to 3017 for eCO2 samples. Using a 97% similarity cut-off, a total of 8899 bacterial OTUs and 897 archaeal OTUs were obtained from the samples collected. To investigate changes in biodiversity, stage variations in Chao1 and Shannon indices were calculated for the microbial communities (Fig. 3A). The Chao1 index calculated for the bacterial group remained relatively constant throughout the experimental period (value between 4100 and 4900), and no significant difference ($p > 0.05$) was observed between the treatment group and the control group. The Shannon index exhibited a similar trend, and the average indices were 10.58 and 11.51 for aCO_2 and eCO_2 samples, respectively. No significant differences ($P > 0.05$) were seen in the Shannon diversity index between aCO_2 and eCO_2 samples.

In the archaeal biosphere, an up-and-down data pattern was observed throughout the entire sampling period. Compared with the bacteria, the α-diversity was much lower (only one-twentieth that of the bacteria). The highest Chao1 diversity index appeared at the beginning (0^{th} week) (the value was 200), and the lowest value was observed after 1week (the value was 90) (Fig. 3B). However, the Chao1 index did not show obvious changes between experimental group and control group at the same time-point. Similarly, the Shannon index was also unaffected by the increased CO_2 level, in spite of some fluctuation existing.

The beta-diversity patterns of the microbial communities (among-sample differences in OTU composition) were shown in supplementary information (Fig. S1), in which the color intensity shows the similarity between the existing species. For both bacteria and archaea, no significant dissimilarity were observed between the experimental group and the control group at different timepoints. Some overlap and interweave distribution were observed in the test samples.

3.4. Rhizosphere microbial community composition

From Fig. 4A, we can see that *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were the most abundant phyla in the total bacterial communities. At the family level, the dominant bacterial clades (top ten) in samples were *Photobacterium*, *Bacillus*, *Microbulbifer*, *Planctomyces*, *Desulfococcus*, *Rhodoplanes*, *Vibrio*, *Alicyclobacillus*, *Desulfovibrio*, *and Methylophaga*, which contributed > 70% of the total quality reads (Fig. 4B). Among them, some taxa displayed obvious

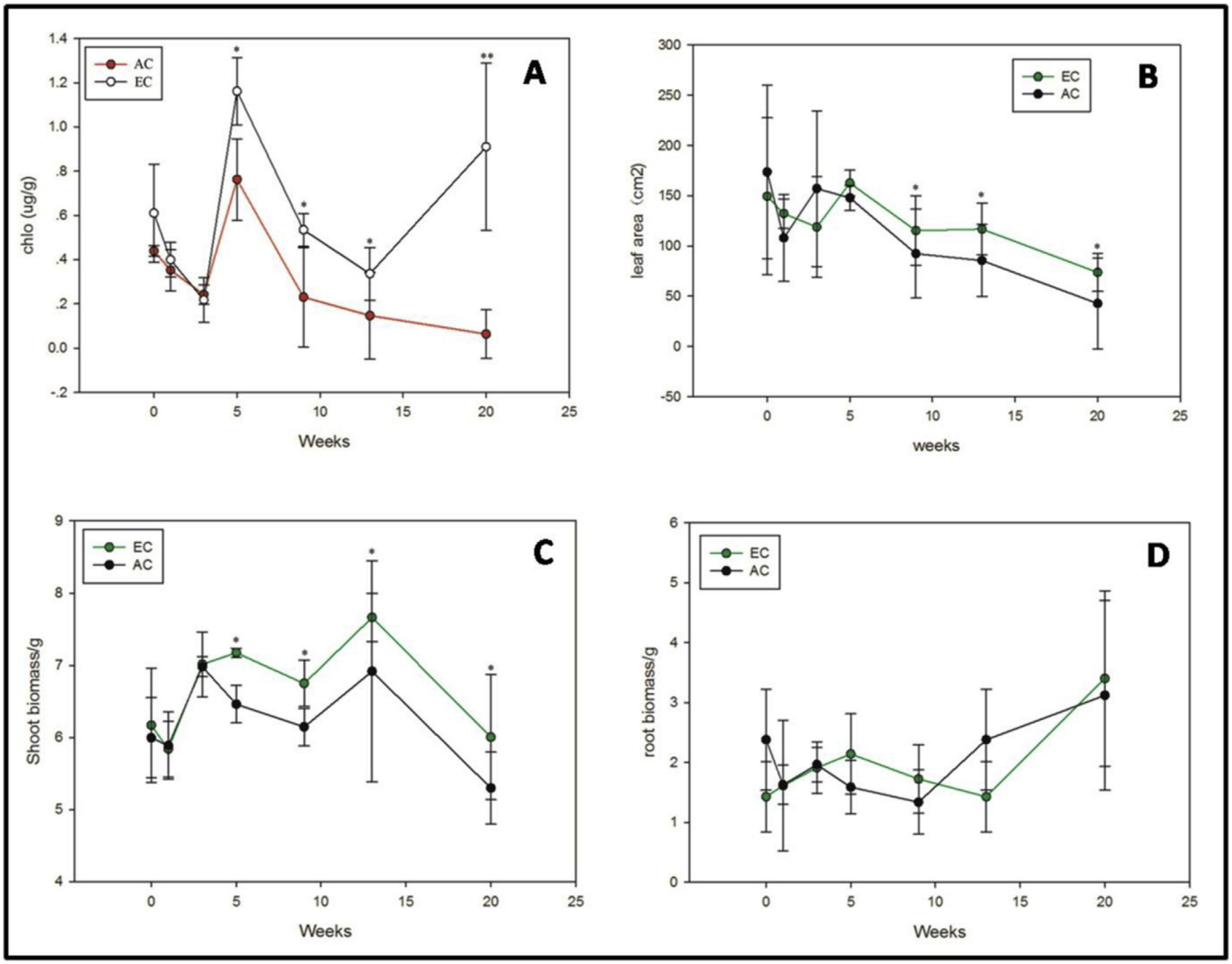

Fig. 2. The *K. obovata* response parameters under eCO_2 treatment during the experimental process. A) ChII *a* level; B) leaf area (cm^2); C) shoot mass; and D) root biomass. Data are shown as the mean ± SD (n = 3); *, statistically different from the control at the P < 0.05 level.

variations during the experimental period. *Photobacterium* gradually increased during the pre-stage, reached maximum proportional abundance at the 1st week (nearly 30%), and then declined with time. Similar to *Photobacterium*, *Bacillus* increased during the CO_2-exposure stages, reached highest proportional abundance at the 5th week, and decreased subsequently. *Microbulbifer* was the third most proportionally abundant genus, and showed an overall decline as the CO_2 exposure progressed. *Planctomyces* and *Desulfococcus* exhibited gradually increasing and decreasing patterns with experimental progression, respectively. However, surprisingly, there were few differences in the relative abundance of taxa among the stages, the main clade, or subclades, and the bacterial community structure remained rather stable. In addition, the profiles of the main colonies of both the experimental group and the control from different time points were very similar; no significant differences were observed between the experimental group and control group during the whole CO_2 exposure process.

Compared with bacteria, relatively fewer archaea were detected. The ratio of archaea was about 8–10% of the total prokaryotic community. The sequences obtained in this study primarily fell within the *Crenarchaeota*, *Parvarchaeota*, and Marine Group II *Euryarchaeota* (Fig. 4C). Among the most abundant OTUs (genus level), > 50% belonged to ammonia-oxidizing archaea (AOA) (*Nitrososphaera*, *Nitrosopumilus*, and *Nitrosotales*); 15–25% belonged to methanogenic species (*Methanoseata*, *Methanosarcina*, *Methanolobus*, and *Methanobacterium*); 3–13% belonged to *Haladaptatus*; and 11–32% belonged to other genera (*Halogranum*, *Cenarchaeum*, *Natronomonas*, and un-classified) (Fig. 4D). Some significant differences were observed between the aCO_2 and eCO_2 groups. The proportion of *Nitrososphaera* rapidly decreased from 40 to 50% (0^{th} week) to only 10% (20th week). Conversely, another AOA member (*Nitrosopumilus*) increased gradually from the pre-CO_2 exposure period to the exponential terminal stages, and its relative abundance increased 2–3 times. Like *Nitrosopumilus*, the proportion of *Nitrosotales* was significantly related to the eCO_2 condition, and the abundance of members of this genus showed significant enrichment at the post-CO_2 exposure stage (20th week) ($P < 0.05$). Methanogenic archaea were present during the entire experimental period. Compared with the aCO_2 group, the proportional abundance of these taxa greatly increased among the eCO_2 group and comprised 30–45% of the total archaeal community (Fig. 4D).

3.5. Correlation of microbial community structure with environmental factors

Canonical correspondence analysis (CCA) was used to examine the environmental factors driving the spatial distribution of microbial diversity and the respective contributions of each environmental variable

Table 1
Soil biogeochemical characters after eCO_2 exposure. Data are shown as the mean ± SE (n = 3). The parameters including pH, salinity, NH_4^+ (μg N/g soil), NO_3^- (μg N/g soil), TC (total carbon, %), TN (total nitrogen, %), C/N radio and MBC (microbial biomass carbon, μg C/g soil).

	AC group (ambient CO_2)							EC group (elevated CO_2)						
	pH	Salinity	NH_4^+	NO_3^-	TC	TN	C:N	MBC	pH	Salinity	NH_4^+	NO_3^-	TC	TN	C:N	MBC
0 week	5.65 ± 0.38	12.0 ± 1.0	0.62 ± 0.06	0.63 ± 0.07	1.55 ± 0.19	0.15 ± 0.01	9.9 ± 0.83	150.2 ± 21.46	5.62 ± 0.52	12.1 ± 0.86	0.61 ± 0.1	0.68 ± 0.09	1.65 ± 0.18	0.16 ± 0.02	10.1 ± 1.12	150.3 ± 18.79
1 week	6.01 ± 0.40	11.8 ± 0.98	2.01 ± 0.20	1.12 ± 0.12	1.66 ± 0.21	0.16 ± 0.01	10.3 ± 0.86	90.3 ± 12.9	6.05 ± 0.50	12.0 ± 0.86	2.05 ± 0.14	1.62 ± 0.20	1.75 ± 0.19	0.17 ± 0.02	9.9 ± 1.1	95.8 ± 11.98
3 weeks	6.22 ± 0.41	11.7 ± 0.99	1.15 ± 0.12	0.40 ± 0.04	1.58 ± 0.20	0.15 ± 0.01	10.0 ± 0.83	62.5 ± 8.93	6.35 ± 0.47	13.3 ± 0.95	1.88 ± 0.13	0.51 ± 0.06	1.70 ± 0.19	0.16 ± 0.02	10.3 ± 1.14	100.5 ± 12.56
5 weeks	5.85 ± 0.39	13.5 ± 1.13	0.78 ± 0.08	0.11 ± 0.01	1.56 ± 0.20	0.14 ± 0.01	10.4 ± 0.87	195.2 ± 27.89	6.05 ± 0.45	13.5 ± 0.96	1.02 ± 0.07	0.11 ± 0.01	1.56 ± 0.17	0.14 ± 0.01	10.4 ± 1.16	250.8 ± 18.58
9 weeks	6.41 ± 0.43	14.2 ± 1.18	1.13 ± 0.11	0.12 ± 0.01	2.01 ± 0.25	0.18 ± 0.02	10.7 ± 0.89	275.6 ± 39.37	6.76 ± 0.56	14.5 ± 1.04	0.95 ± 0.06	0.11 ± 0.01	1.78 ± 0.20	0.16 ± 0.02	10.6 ± 1.18	295.9 ± 34.49
13 weeks	6.25 ± 0.42	12.3 ± 1.03	0.60 ± 0.06	0.14 ± 0.02	1.60 ± 0.20	0.16 ± 0.01	10.1 ± 0.84	149.5 ± 21.36	6.31 ± 0.55	14.0 ± 1.0	1.03 ± 0.07	0.12 ± 0.02	1.62 ± 0.18	0.16 ± 0.02	10.5 ± 1.17	225.4 ± 28.18
20 weeks	6.35 ± 0.42	14.3 ± 1.09	0.72 ± 0.07	0.22 ± 0.02	1.82 ± 0.23	0.17 ± 0.02	11.5 ± 0.96	170.2 ± 24.31	6.27 ± 0.50	13.5 ± 0.96	0.62 ± 0.01	0.51 ± 0.06	1.53 ± 0.17	0.15 ± 0.01	10.7 ± 1.19	180.7 ± 18.84

to the total explained variance. Among the environmental factors, NH_4^+, NO_3^-, and C/N ratio contributed most to the variance in bacterial communities (Fig. 5A), whereas the three strongest determinants of community structure of archaea were C/N ratio, salinity and TN (Fig. 5B). Among the host-related parameters, MBC had the greatest influence on the community diversity followed by ChII *a* concentration and shoot biomass. There were no obvious negative or positive correlations between population dynamics and pH value.

A variation partitioning analysis was carried out to partition the contributions of detected parameters. Results of this analysis showed that environmental (including pH, salinity), nutrient-related (including NH_4^+, NO_3^-, TC, TN, and C/N ratio), and host-related (including MBC, ChII *a*, shoot mass, and root biomass) parameters explained 31.0% of microbial community variation in the OTU data (Fig. 6), indicating that they were significant factors in determining microbial composition. Independently, environment, nutrients, and host were able to explain 11.9% ($P = 0.01$), 12.7% ($P = 0.05$), and 6.4% ($P = 0.05$) of the total variation observed, respectively. Interactions between environment and nutrients, environment and host, as well as nutrients and host explained 2.9%, 1.8%, and 1.6% of variation, respectively. About 69.0% of the microbial community variation in OTU data was not explained by these parameters, indicating that other biotic and/or abiotic factors contribute to this process.

3.6. Microbial functions

The ability of culturable microbes to metabolize carbon compounds following the eCO_2 treatment was evaluated at the 0th, 5th, and 20th weeks. From Fig. 7A, we can see that no significant carbon metabolic differences were present before the 700 ppm CO_2 exposure. Interestingly, after 5 weeks, a significantly higher ACWD value was observed compared with the control ($P < 0.05$) (Fig. 7B). In addition, the ACWD measured under combined eCO_2 was further increased compared with that measured under control conditions at the 20th week ($P < 0.05$) (Fig. 7C). The speed of utilization of carbon sources was faster under eCO_2 than in the control group.

To determine the types of carbon source involved, we measured the carbon metabolic patterns at the terminal point (20th week). For the control group, Fig. 8A shows that root microbes preferred carbohydrates, amino acids, and carboxylic acids as substrates, which coincidentally are also the major carbon sources available for bacteria. However, significant change was appeared in the eCO_2 treatment, the results showed that carbon source utilization occurred in the order amino acids > carbohydrates > polymers > carboxylic acids > amines phenolic acids (Fig. 8B).

4. Discussion

Our study examined eCO_2 effects on mangroves, and found that ChII *a* level, leaf area, and shoot mass were increased after 3–5 weeks (Fig. 2A–C). The increased ChII *a* content indicates that mangrove photosynthetic rates were increased under a CO_2-enriched atmosphere (Takahashi, 1978). In addition, the positive responses of leaf area and shoot mass may be related to an eCO_2-induced increase in the soil water content through reduced stomatal conductance (Kastl et al., 2015) and evaporation-transpiration (Grant et al., 2008). However, no stimulation of root biomass was observed under atmospheric CO_2 enrichment (Fig. 2D). A possible reason was the short incubation time period with eCO_2, which did not allow the plant to fully adapt to the changed environment. Under our experimental condition, root growth was not favored under eCO_2 because increased CO_2 may not have affected soil organisms indirectly through increased availability of labile C through exudation (Phillips et al., 2009) or increased carbon flux from plants to the soil (Smith et al., 2010).

Among the soil parameters (pH, salinity, NH_4^+, NO_3^-, TC, TN, C/N ratio), no significant differences were observed between the treatment

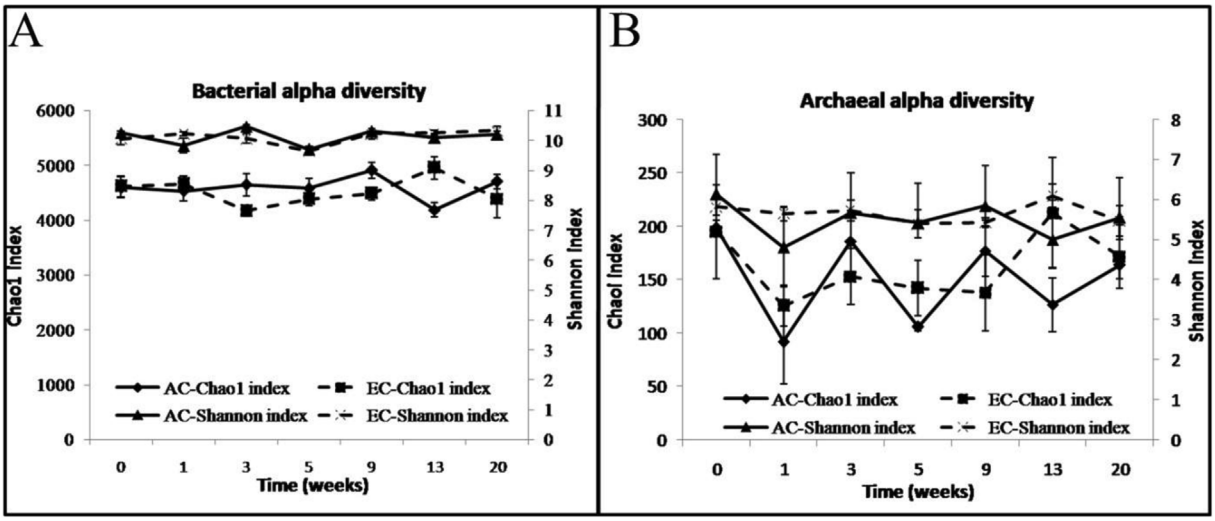

Fig. 3. The α-diversity indexes of the test samples collected from the aCO_2 group and the eCO_2 group at a 0.03 distance level. A) Chao1 index; and B) Shannon index.

group and control group (Table 1). These results are in accordance with those of Insam et al. (1999), who found that elevated CO_2 concentration did not have significant impacts on the content of soil organic carbon, TN, or DOC in rhizosphere soils. However, some research has demonstrated that eCO_2 can alter TC, TN, and C/N ratio in land or aquatic plants (Ainsworth et al., 2007; Mata et al., 2013; Buchner et al., 2015). The inconsistencies may arise because usually the sole environmental manipulation is atmospheric CO_2 concentration, while the response of soil ecosystems to eCO_2 conditions has been shown to be highly dependent on interactions among fertility, water, and temperature (Zak et al., 2000; Groenigenet al., 2006). For the MBC, we noted some predictable tendency: a slight rise in CO_2-treated group, which is probably attributable to higher carbon assimilation rates due to greater carboxylation efficiency of Rubisco (this enzyme catalyzes the initial fixation of CO_2) (Cruz et al., 2006).

Besides the plant and soil characters, we hypothesized that the eCO_2

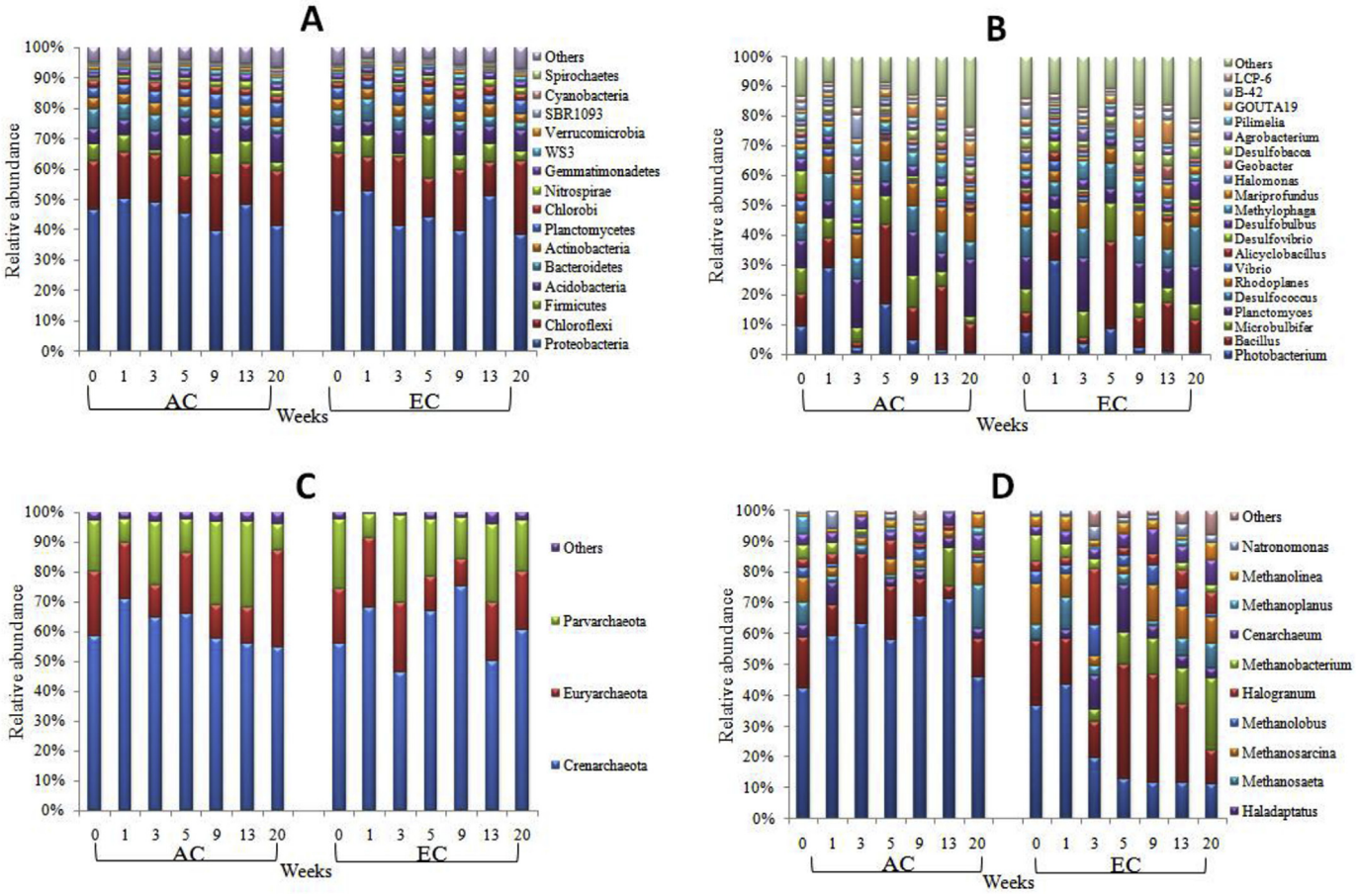

Fig. 4. Microbial communities in soil samples exposed to CO_2 treatments. A) and B): bacterial communities at the phylum and family levels, respectively. C) and D): archaeal composition at the phylum and family levels, respectively.

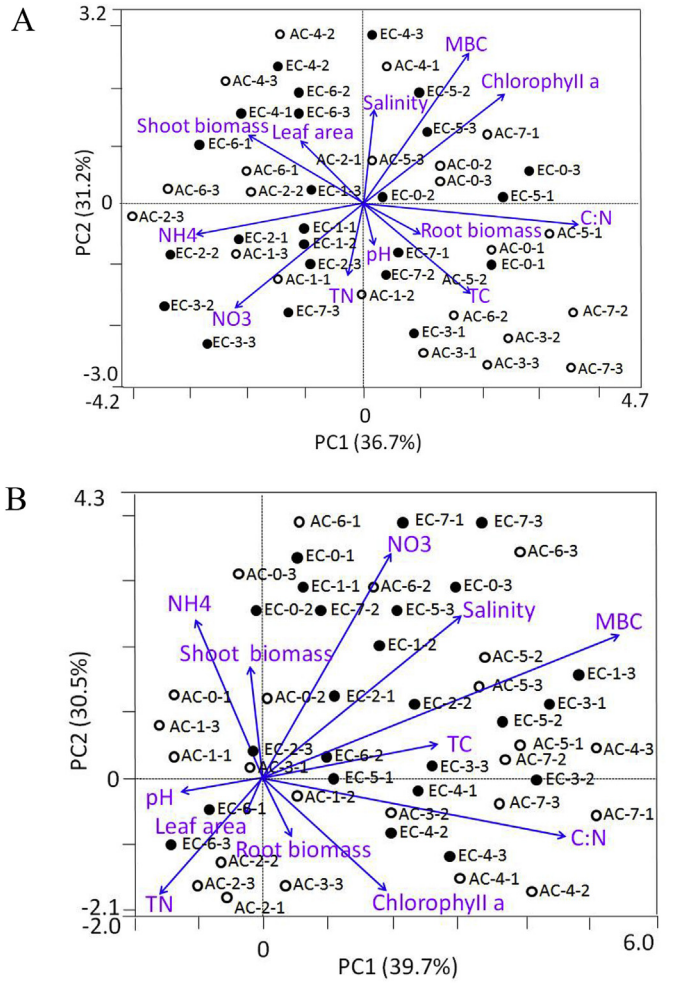

Fig. 5. Canonical correspondence analysis (CCA) based on OTUs and environmental parameters during the experiment. A) bacteria, B) archaea. AC and EC refer to samples from seven different time points (0, 1, 3, 5, 9, 13, and 20 weeks), e.g., EC1-1, EC1-2, and EC1-3 represent three parallel samples for the first sampling of the eCO_2 group, and AC1-1, AC1-2, and AC1-3 represent the first time-point for the aCO_2 group. The rest can be done in the same manner. Environmental factors are represented by arrows, and the length of the arrow represents the degree of correlation between the environmental factor and the community structure. The angle between the principal axis and the major axis represents the correlation between the environmental factor and the axis.

would cause a change in rhizosphere microbial diversity. In our study, however, eCO_2 did not change the α-diversity (Chao1 and Shannon index) and β-diversity significantly (Fig. 3AB, Fig. S1). Similar results have been observed in terrestrial soil communities. In experimental grassland plots exposed to 650 ppm of CO_2, there was no detectable increase in the species richness of the communities even after 5 years of exposure (Klamer et al., 2002). The lack of change in the overall microbial diversity may also reflect the adaptive nature of these communities to environmental perturbations, including atmospheric CO_2. Fossil evidence indicates that microbes have been present on Earth for billions of years and in the past few hundred millions of years have experienced a wide range of CO_2 fluctuation from current levels to upwards of 25 times present atmospheric levels during the early Cambrian period (542 Ma) (Sheldon, 2006). In addition, another reason may be due to the redundancy in the microbial community structure analyzed in our study. Yin et al. (2000) reported that soil bacterial communities exhibited a degree of redundancy, in that a number of different component functional groups were capable of responding to ecological opportunities. The present study allows us to speculate that *K. candel* microorganisms have a certain buffering adaptability under short-term CO_2 exposure, and their diversity has limit affected by greenhouse gases.

Similar with the biodiversity results, some studies have shown no alteration of microbial community composition by elevated CO_2 concentrations. Zak et al. (2003) found no significant response in microbial community composition in soil under eCO_2 conditions. Klamer et al. (2002) also reported that elevated CO_2 caused only subtle changes in Gram-negative bacteria and actinomycetes. In an artificial tropical ecosystem, Insam et al. (1999) further pointed out that elevated CO_2 did not affect the shift in bacterial community by employing phospholipid fatty acid analysis (PLFA) patterns and community level physiological profiles (CLPP). Similar to these reports, we did not find significant differences between the experimental group and the control group (Fig. 4AB). The absence of significant effects from eCO_2 alone on the bacterial community may be due to the variance in other biogeochemical parameters induced by the eCO_2 (Andersen et al., 2013). Ge et al. (2010) reported that spatial factors, which are caused by various parameters including plant type, have a greater impact in determining the bacterial community than eCO_2 by itself. In addition, some other factors also affect the response of the bacterial community, such as soil feature, local environment, and even the plant age and plant stage. It can be inferred that the bacterial response to interactive effects of CO_2 may represent a more complex scenario.

Unlike the bacteria, a different picture emerged in the archaeal community (Fig. 4D). The majority of the archaeal community members were assumed to be AOA members (ammonia oxidizing archaea) and methanogenic archaea, which suggested a high potential CH_4 emission from this wetland micro-system (Nelson et al., 2008). The

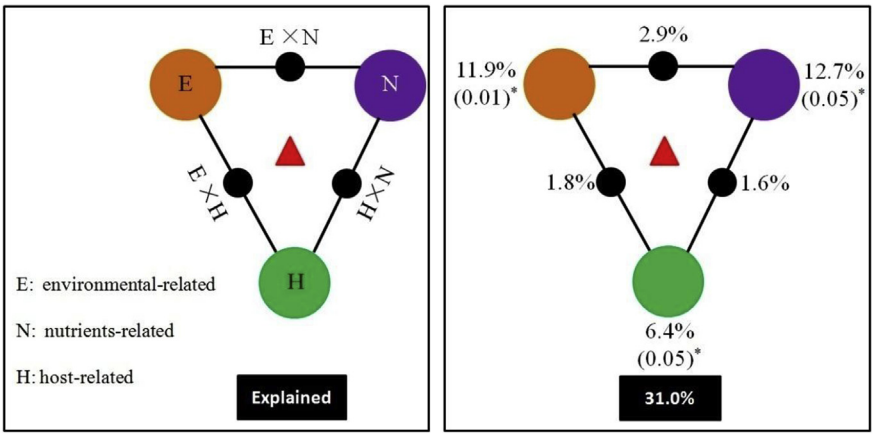

Fig. 6. Variation partitioning analysis of microbial distribution explained by environmental factors. A) general outline; B), test environmental parameters, including environmental-, nutrient-, and host-related factors. Each diagram represents the variation partitioned into the relative effects of each factor or combination of factors, in which area is proportional to the respective percentages of variation explained. The edges of the triangles represent the variation explained by each factor alone. The sides of the triangles represent interactions of any two factors, and the middle parts of the triangles represent interactions of all parameters.

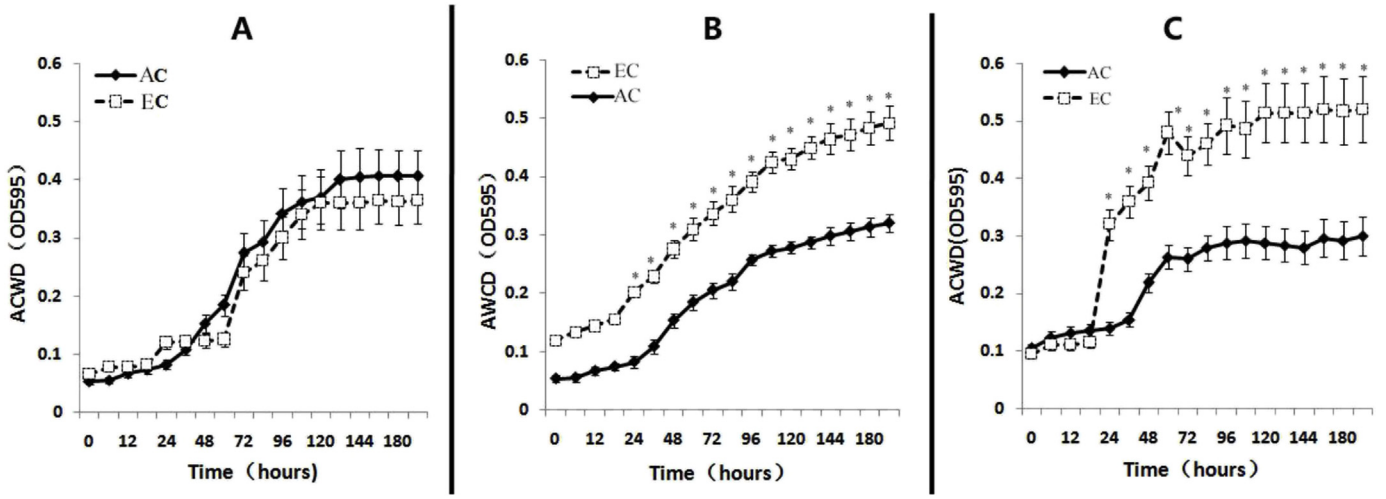

Fig. 7. Average well color development (AWCD) of microorganisms as a result of carbon utilization between the experimental group and the control group. A), B), and C) represent the 0th, 5th, and 20th weeks, respectively.

methanogenic archaea showed a significant response to eCO_2, which was due to their ability to utilize CO_2 directly. The significant increase observed in the methanogenic archaea suggests the low stability and dynamic response of this group to external change. Combining this result with our previous observation (Yin et al., 2017) of a decrease in archaeal abundance under eCO_2, we can infer that CO_2 concentration has a large impact on the archaeal community in the mangrove ecosystem.

Among the AOA species (especially *Nitrosopumilus* and *Nitrosotales*), which showed a significant increase under eCO_2 conditions (Fig. 4D). It has been proposed that for AOA, the type of organic matter, particularly labile organic carbon, may play a role in increasing AOA abundance (Wessén et al., 2010). In this study, we observed slightly increased TC (although not significantly) by eCO_2, which partly explains the AOA increase. The AOA community change in response to eCO_2 was also supported by AOB (ammonia-oxidizing bacteria, such as *Nitrospira*). We found *Nitrospira* was significantly increased after CO_2 exposure. The response of AOA and AOB indicates that the ammonia in the soil is oxidized and decomposed to nitrate-nitrogen (Shinohara et al., 2012). In contrast with *Nitrosopumilus* and *Nitrosotales*, another AOA member (*Nitrososphaera*) was decreased in the CO_2 exposure environment. It has been reported that some AOA species are less competitive than plants under stress conditions in a subtropical wetland (Chen et al., 2015), and there is a selective effect of the root on the composition of the archaeal community (Llirós et al., 2014). Shen et al. (2013) also reported a negative response of *Nitrososphaera* to eCO_2 in a marsh field. It has been suggested that compared with the C4 plants, C3 plants (such as mangrove plants) are more affected by eCO_2 (Sage and Kubien, 2003; Barbehenn et al., 2004).

Among the environmental parameters analyzed, we found that

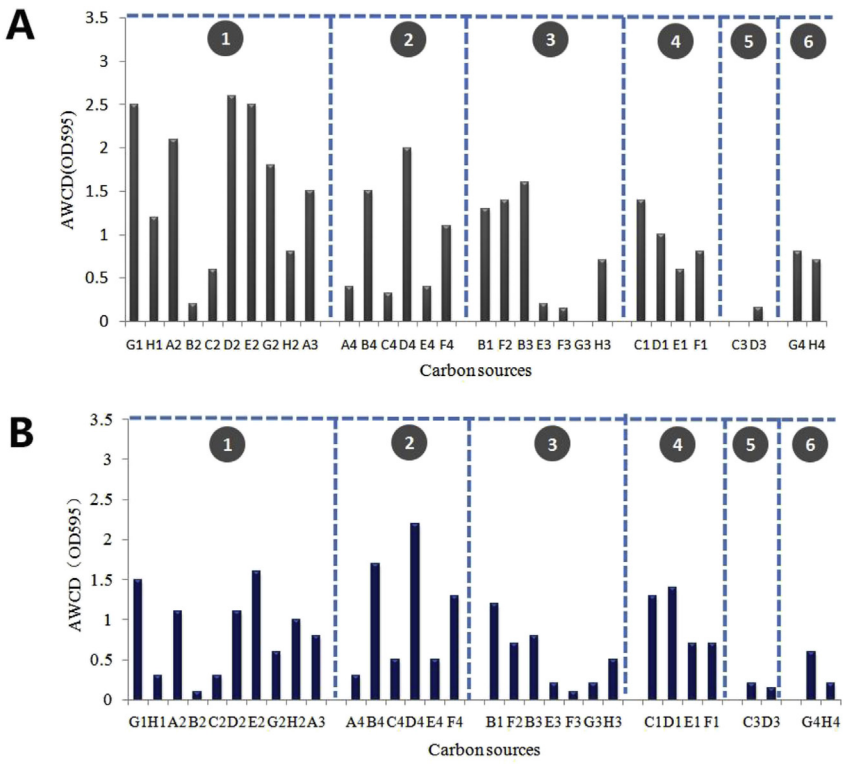

Fig. 8. Metabolic fingerprint of carbon utilization profiles of root microorganisms between the experimental group and the control group. Results are taken from the terminal stage (20th week) of the experiment as an example. A total of 33 carbon sources are depicted. Group 1 (G1-A3) represents carbohydrates, Group 2 (A4-F4) represents amino acids, Group 3 (B1-H3) represents carboxylic acids, Group 4 (C1-F1) represents polymers, Group 5 (C3-D3) represents phenolic acids, and Group 6 (G4-H4) represents amines.

salinity was strongly correlated with bacterial structure, and also influenced archaeal communities (Fig. 5AB). These results indicate that osmotic pressure was the major abiotic force shaping microbial community structure in mangrove plants, and are consistent with past studies that documented a pronounced impact of salinity on microbial composition. For example, Lozupone and Knight (2007) demonstrated that salinity is the major environmental determinant of microbial communities rather than temperature, pH, or other physical and chemical factors. The salinity differences could explain why the functional structure of sediment microbial communities in a fringing mangrove forest was different (Ikenaga et al., 2010). Salinity was previously shown to be a principal driving force of archaeal community patterns at the global scale (Auguet et al., 2010); these results demonstrate the importance of salinity in determining archaeal community structure at local scales.

In addition to physical factors, N and C are two key chemical elements that influence microbial communities through their effects on substance utilization and growth. The acquisition and efficiency of C/N are tightly coupled to specific nutrient stoichiometry, which subsequently governs the succession of dominant taxa. In this work, we observed that concentrations of MBC, NO_3^-, NH_4^+ and C/N radio were obvious related to bacteria and archaea. These results support prior findings in which the root microbial community was regulated by nutrient availability, which contributes to bottom-up control over both host and microbial populations (Surbeck et al., 2010).

The environmental data were further analyzed to determine which parameters drive community variability. These analyses included environmental-, nutrient-, and host-related parameters as explanatory variables. These factors were able to independently explain 11.9%, 12.7%, and 6.4% of the total observed variations, respectively (Fig. 6). Abiotic (natural conditions and nutrient availability) and biotic (copresence or exclusion) factors have long been regarded as the two primary drivers shaping the distribution of microbial communities; the former has been considered to have a stronger effect (Singh et al., 2009). However, Limamendez et al. (2015) reported that environmental factors contributed to just 18% of community variation of microbes at the global ocean scale. Similarly, in this study, just 31.0% of microbial community variation in the OTU data was explained by the above-mentioned parameters, indicating that other abiotic factors not sampled (such as DOC, DOM, or ROS abundance) or biotic parameters (e.g., some uncovered community interactions) may be important drivers of community structure. These findings, however, may also indicate that abiotic factors have a more limited effect on community structure than has been assumed. In future work, more physical-chemical parameters and longer-term dynamic detection need to be carried out in order to obtain more comprehensive/accurate results.

As for microbial function, from Figs. 7 and 8, we can see that the utilization speed and type of carbon source changed under eCO_2. The acceptable explanation of metabolic ability changes might be the active and effective carbon source supplemented by root exudates. The Biolog profile indicated that the carbon sources such as amino acids, carbohydrates, and carboxylic acids, which were similar to the root exudate components, had a higher utilization ratio in the CO_2-treated soil. The Biolog data also provided potential carbon utilization activities for fast-growing heterotrophic bacteria. In general, Gram-negative (G^-) bacteria are fast-growing species that tend to utilize simple C substrates, whereas Gram-positive (G^+) bacteria are slower-growing species that tend to specialize on more complex C substrates (Wickings et al., 2012; Creamer et al., 2015). A change in the relative abundance of G^+ or G^- bacteria may result in a shift in the decomposition of complex C in the soil, which consequently could influence soil C storage (Balser and Firestone, 2005). Based on this theory, we speculated that eCO_2 probably altered the G^+/G-bacteria ratio, in spite of the whole microbial community is relatively stable. This is the first report eCO_2 influence on function of rhizosphere bacteria in mangrove plant. In the next step, we plan to focus on metagenomic or metatranscriptomic to reveal the molecular mechanisms of root microorganisms under CO_2 exposure.

5. Conclusions

Our results indicate that eCO_2 has limited ability to affect bacterial communities, while it has a more obvious affect on the archaeal community; and different species show different responses to eCO_2. The mangrove rhizosphere microbial communities have some buffering ability in response to short-term CO_2 increase whereby the metabolic efficiency of carbon sources is changed. This study helps us to better understand the structural inflexibility and functional plasticity of the rhizosphere microbiome in mangrove plants facing a changing environment. To better understand the impacts of global climate change on microbial communities and the biological feedback in wetland ecosystem, further studies involving *in situ* long-term monitoring are necessary. In addition, alternative approaches using stable-isotope-probing methods and Omcis technology are also desirable to understand the functionality of active microbial communities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.marenvres.2018.07.013.

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